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Cigarette smoke decreases MARCO expression in macrophages: Implication in *Mycoplasma pneumoniae* infection☆

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Summary
Bacterial infections including *Mycoplasma pneumoniae* (Mp) are a major cause of exacerbations in chronic obstructive pulmonary disease (COPD). Cigarette smoke (CS) is the leading cause of COPD, and affects the function of alveolar macrophages that act as the first line of defense against the invading respiratory pathogens. Macrophages express a transmembrane receptor called *ma*crophage *r*eceptor with *co*llagenous structure (MARCO) that is involved in the clearance of microorganisms. Whether CS down-regulates MARCO and eventually decreases the clearance of Mp has not been investigated. We utilized human monocytic cell line (THP-1)-derived macrophages to examine the effects of CS extract (CSE) on MARCO expression and Mp growth. Specifically, macrophages were pre-exposed to CSE for 6 h, and then infected with or without Mp for 2 h. MARCO was examined at both mRNA and protein levels by using real-time PCR and immunofluorescent staining, respectively. Mp in the supernatants was quantified by quantitative culture. In addition, a neutralizing MARCO antibody was added to macrophages to test if blockade of MARCO impaired Mp clearance. We found that CSE significantly decreased MARCO expression in a dose-dependant manner at 6 h post-CSE. Mp levels in CSE-treated cells were higher than those in non-CSE-treated cells, indicating a decreased pathogen clearance. Additionally, neutralizing MARCO in macrophages markedly increased Mp levels. Our results indicate that cigarette smoke exposure down-regulates MARCO expression in macrophages, which may be in part responsible for impaired bacterial (e.g., Mp) clearance.

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Introduction

Bacterial infections continue to pose a huge burden on COPD patients. Many species of bacteria including nontypeable *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae* and *Chlamydia pneumoniae* have been detected in COPD lungs.[1] In addition, *Mycoplasma pneumoniae* (Mp) was found to be one of the common (14%) pathogens in COPD patients.[2,3] One of the fundamental research questions is: why are COPD patients more susceptible to bacterial infections than normal human subjects? In other words, is there any deficiency of host innate immune responses to an invading pathogen in COPD patients?

Cigarette smoke (CS), a leading cause of COPD, may predispose COPD patients to bacterial infections, and exaggerate inflammatory responses by various mechanisms. Mucosal hypertrophy, increased mucus production, decreased tracheal mucosal velocity, increased lung neutrophils and production of IL-8 (a chemoattractant for neutrophils), and increased expression of adhesion molecules like E-selectin are some of the effects that CS exerts in COPD patients with bacterial infections.[4–10] Further exploration into the relationship between CS and COPD reveals that CS is also involved in the activation of alveolar macrophages that contribute to the pathogenesis of COPD by releasing various inflammatory mediators such as LTB_4, PGE_2, IL-8, TNF-α, TGF-β, superoxide anions, iNOS and proteases.[11–18]

The macrophages utilize several mechanisms contributing to bacterial clearance. These include superoxide anions, nitric oxide and cell receptor (e.g., Toll-like receptors)-mediated production of inflammatory cytokines involved in the recruitment of inflammatory cells (e.g., neutrophils). The role of cell surface receptors in macrophage host defense functions has been under intensive investigation. Macrophages have a very active plasma membrane expressing high densities of different types of receptors like immumoglobulin receptors, complement receptors, mannose receptors and TLRs. Among them, scavenger receptors (SRs) have been proposed to be critical in normal lung defense. SRs are transmembrane proteins on the surface of macrophages and exhibit high-affinity binding to various ligands. These receptors are divided into classes A, B and C based on their structure.[19,20] Recently included within class A of SRs is a novel receptor, macrophage receptor with collagenous structure (MARCO). MARCO was initially discovered in mice using a human type 3-collagen cDNA probe, and was later identified as a receptor binding to bacteria.[21–23]

In the current study, we proposed that MARCO is involved in the clearance of Mp. We further hypothesized that CS would inhibit MARCO expression, thus impairing bacterial clearance. To test our hypothesis, we used a human macrophage cell culture system to examine the interplay between CS exposure, MARCO and Mp.

Materials and methods

Cigarette smoke extract (CSE) preparation

The CSE was prepared as previously described.[24] Briefly, one 83 mm unfiltered research grade 2R1 cigarette was combusted with the use of a peristaltic pump into 25 ml of sterile RPMI-1640 medium that did not contain any serum (e.g., fetal bovine serum). The smoke-saturated RPMI-1640 medium was filter-sterilized through a 0.22-μm filter, and the resulting preparation was considered as 100% CSE which was used within 30 min of preparation by diluting it into macrophage culture medium to perform the cell culture experiments.

Mycoplasma pneumoniae (Mp) preparation

Mp (strain FH; ATCC 15531) was grown in SP-4 broth for 5 days at 37 °C in T-75 flasks, scraped off the flasks, and was then passed through a 26-gauge needle to prevent Mp self-aggregation. Mp was then plated on PPLO plates to quantify colony forming unit (CFU). The remainder of Mp at passage 4 was frozen at −80 °C, and subsequently used for cell culture experiments. On the infection day, frozen Mp aliquots were thawed, spun, resuspended in SP-4 broth, and incubated for 2 h at 37 °C. Mp was then spun at 6000 rpm for 5 min and resuspended in cell culture media to yield the indicated concentrations for infecting cells.[25]

Cell culture

The human monocytic cell line THP-1 cells were obtained from the American Type Culture Collection (ATCC, product number TIB-202), and the cells in RPMI-1640 medium with 10% fetal bovine serum (FBS, GIBCO, Invitrogen) were plated on 12-well plates with 4×10^5 cells/well. To these cells 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) was added overnight (16 h) to induce THP-1 cell differentiation into macrophages. The next day, PMA along with non-adherent cells, was washed off three times with PBS, resulting in adherent macrophages on the plates.

Preliminary experiments ($n = 3$) were performed to optimize the impact of duration of CSE exposure on MARCO expression. THP-1 cell-derived macrophages were exposed to various concentrations of CSE (5, 10 and 20%) for 2, 6, 24 and 48 h. Cells were collected for measuring MARCO mRNA expression by real-time PCR. The time point, which yielded the most significant and consistent reduction in MARCO expression, was seen at 6 h (about 3-fold decrease with 20% CSE, Table 1). At 2, 24 and 48 h, there was no significant change in MARCO expression with all the three concentrations of CSE used. Therefore, CSE exposure for 6 h was chosen as the optimal condition for all the experiments described in Results section.

To validate the use of 20% CSE in our cell culture experiments, nicotine content in 20% CSE preparation was

Table 1 Time course study of MARCO mRNA expression in THP-1 cell-derived macrophages ($n = 3$)

Time points (h)	MARCO mRNA relative level − control (no CSE)	MARCO mRNA relative level − 20% CSE
2	1.8 ± 1.2	1.5 ± 2.8
6	3.0 ± 1.5	1.0 ± 2.3
24	5.9 ± 1.5	6.1 ± 2.3
48	5.6 ± 1.5	5.8 ± 1.8

measured at 3.4 ± 0.05 μg/ml by using the HPLC method, which is relevant to the physiologic levels of nicotine in human smokers' lungs based on the following facts: (1) 82—92% of nicotine in lung epithelial lining fluid (ELF) diffuses into the blood stream[26]; (2) The volume of ELF in human smokers is 2.4 ± 1.4 ml,[27] which is about 1000-fold lower than that of serum (average = 2750 ml for an adult); and (3) Blood nicotine concentration in human smokers is 6—52 μg/L (or 0.006—0.052 μg/ml).[26] Therefore, to reach a similar concentration of nicotine between ELF and serum, the initial concentration of nicotine in ELF is most likely 100 to 1000-fold higher than that is finally seen in the serum.

Mp culture in THP-1 cell-derived macrophages

THP-1 cell-derived macrophages were pre-treated with CSE or PBS for 6 h, and then infected with Mp (1 CFU/cell) for 2 h. Immediately after the addition of Mp into THP-1 cell-derived macrophages, the 12-well cell culture plates were gently (200 rpm, 30 s) agitated on a plate shaker to allow Mp to attach to the cells through diffusion. Cell supernatants were collected and serially (10-fold) diluted in SP4 broth with 10 μl of this broth being plated onto PPLO agar plates (Remel, KS, USA). After 7 days of incubation at 37 °C, 5% CO_2, CFU on the PPLO plates was counted under the microscope.

It was worth noting that we chose 2 h of Mp infection since our preliminary experiments ($n = 3$) demonstrated a maximal delay of Mp clearance in THP-1 cell-derived macrophages that were pre-treated with 20% CSE and infected with Mp at 1 CFU/cell. At other time points (4 and 24 h) and doses (5 and 10 CFU/cell) of Mp infection, there was a minimal effect of CSE treatment on Mp clearance. Also, to exclude the possibility of direct killing effects of CSE on Mp, macrophage-free Mp was incubated with or without 20% CSE for 6 h ($n = 6$), followed by culture on PPLO plates. It was found that CFU counts with or without CSE treatment were similar ($71 \pm 8.6 \times 10^4$ vs. $65 \pm 6.7 \times 10^4$ CFU/ml, $p > 0.05$). Thus, 20% CSE alone does not appear to exert any bactericidal activity against Mp. To address whether CSE may affect Mp's ability to bind the macrophages, we performed a CSE washout experiment. After 6 h of incubation, CSE was either rinsed from macrophages or stayed with the cells, and then infected with Mp for 2 h. Our data suggest that Mp levels were similar between macrophages with and without washout of CSE, suggesting that the increased levels of Mp in CSE-pretreated cells may not be caused by CSE-induced impairment of Mp binding to macrophages.

MARCO antibody treatment

Mouse anti-human MARCO antibody (Clone PLK1, mouse IgG_3) was obtained from Hycult Biotechnology (The Netherlands). This antibody was used to inhibit MARCO function in THP-1 cell-derived macrophages, and also to detect MARCO protein expression using immunofluorescent staining. In MARCO blocking experiments, anti-MARCO antibody (0.002—0.2 μg/ml) or matched mouse IgG_3 isotype control was added to THP-1 cell-derived macrophages 1 h prior to Mp infection (1 CFU/cell). After 2 h of Mp infection, supernatants were collected to perform quantitative mycoplasma culture.

Real-time RT-PCR

Total RNA was extracted from THP-1 cell-derived macrophages and treated with DNase 1 (Ambion, Austin, TX, USA) to remove any contaminating genomic DNA. Reverse transcription (RT) was performed using 1 μg of total RNA and random hexamers in a 50 μl reaction according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). The human MARCO primers and probe as shown below were designed using the Primer Express software (Applied Biosystems). Forward primer: 5′-CTGGTGGTCCAAGTTCTGAATCT-3′; reverse primer: 5′-TCAGCCGCCAGAGTGTCA-3′; and probe: 5′-CTCCGGGTCCTGGAGATGTATTTCCTCA-3′. Real-time PCR was performed on the ABI Prism® 7700 sequence detector (Applied Biosystems). The 25 μl PCR contained 30 ng of cDNA, 100 nM fluogenic probe, and 100 nM primers and other components from the TaqMan® RT-PCR kit (Applied Biosystems). GAPDH mRNA was evaluated as an internal positive control. The comparative Ct method was used to calculate the relative mRNA levels of target genes.[28]

MARCO immunofluorescent staining

Immunofluorescent staining was performed to determine MARCO protein expression level after CSE exposure. THP-1 cell-derived macrophages on 8-well chamber slides were treated with or without CSE (5, 10 and 20%) for 6 h, fixed with methanol, and then incubated with the MARCO antibody or mouse IgG_3 (isotype control), followed by FITC-conjugated anti-mouse IgG antibody (Vector Laboratory). Before staining with the relevant antibody, cells were incubated with excess of horse normal serum for 30 min to block nonspecific binding. A Scion image analysis program (National Institutes of Health, Bethesda, MD) was used to measure the mean fluorescent intensity that was then normalized to the total number of cells on the slide.

Statistical analysis

Normally distributed data are presented as mean ± SEM and compared using the matched-pairs *t*-test. Non-normally distributed data are expressed as medians with interquartile (25—75%) ranges and compared using the Wilcoxon rank-sum test. A value of $p < 0.05$ was considered significant.

Results

CSE decreases MARCO mRNA expression

MARCO mRNA expression levels as measured after 6 h of exposure to varying concentrations of CSE are shown in Fig. 1. When compared with the control, CSE at 5, 10 and 20% resulted in a dose-dependent decrease of MARCO mRNA expression. The % change of MARCO mRNA levels over the controls was $74 \pm 25\%$ ($p = 0.06$), $64 \pm 18\%$ ($p = 0.01$), and $54 \pm 29\%$ ($p = 0.03$) for 5, 10 and 20% CSE, respectively. CSE did not affect cell viability as assessed by trypan blue exclusion assay (cell viability, 20% CSE = $98.6 \pm 0.2\%$; control (no CSE) = $94.7 \pm 2.1\%$, $p > 0.05$).

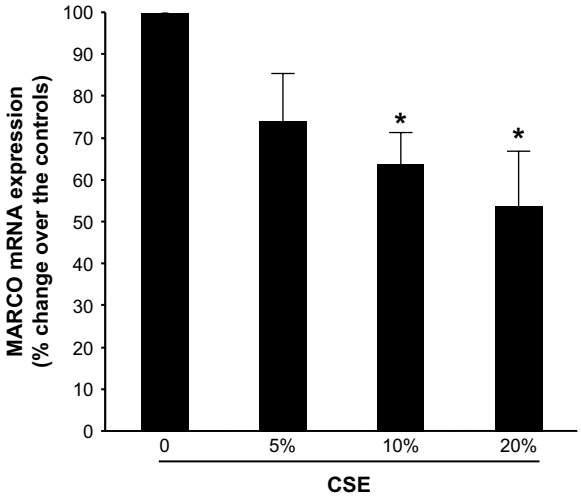

Figure 1 Cigarette smoke extract (CSE) decreases MARCO mRNA expression in THP-1 cell-derived macrophages. Cells were with CSE at various concentrations, or with cell culture medium (control, no CSE) for 6 h. MARCO mRNA expression was determined using real-time PCR, and expressed as % change over the controls. Data are expressed as mean ± SEM ($n = 5$). *$p < 0.05$ compared to controls (no CSE).

CSE inhibits MARCO protein expression

To confirm if inhibition of MARCO mRNA expression by CSE was coupled with protein reduction, immunofluorescent staining was performed to examine MARCO protein. Fig. 2A—D illustrates the changes and pattern of MARCO immunofluorescent staining in THP-1 cell-derived macrophages that were exposed to varying concentrations of CSE for 6 h.

Measurement of MARCO fluorescent intensity on THP-1 cell-derived macrophages with CSE treatment was normalized to the controls (i.e., no CSE exposure) and hence expressed as % change over the controls. Similar to MARCO mRNA data, a dose-dependant decrease in MARCO protein expression was found in macrophages upon 6 h CSE exposure. The % change of MARCO protein over the controls was 99.6 ± 58.3 ($p = 1.0$), 83.8 ± 30.8 ($p = 1.0$), and 68.1 ± 15.5 ($p = 0.014$) for 5, 10 and 20% CSE, respectively.

CSE impairs Mp clearance in macrophages

As shown in Fig. 3, Mp levels in the supernatants of THP-1 cell-derived macrophages with 6 h of 20% CSE pre-treatment were significantly ($p = 0.008$) higher than those without CSE treatment in all six independent experiments.

MARCO blockade increases Mp levels in the supernatants of macrophages

Blocking MARCO with a neutralizing antibody (0.002, 0.02, 0.2 μg/ml) ($n = 4$) resulted in an increase in Mp counts in the supernatants of THP-1 cell-derived macrophages (Fig. 4). MARCO antibody-induced % increase in Mp levels over the controls was 17.2 ± 3.4 ($p = 0.7$), 29.0 ± 3.4 ($p = 0.03$), and 29.0 ± 1.7 ($p = 0.004$) for MARCO antibody at 0.002, 0.02 and 0.2 μg/ml, respectively. MARCO

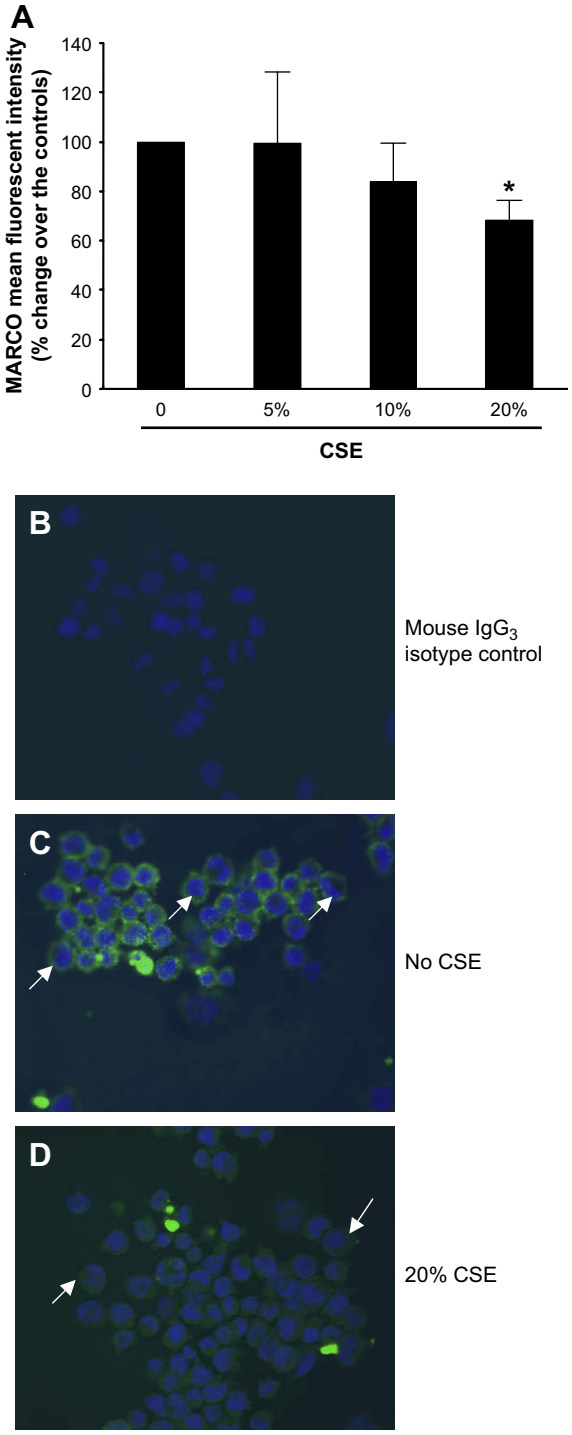

Figure 2 Cigarette smoke extract (CSE) decreases MARCO protein expression in THP-1 cell-derived macrophages in a dose-dependent manner. Cells were with CSE at various concentrations, or with cell culture medium (control, no CSE) for 6 h. MARCO protein expression on macrophages was determined using immunofluorescent staining. (A) Quantitative data of MARCO immunofluorescent staining are expressed as mean ± SEM ($n = 4$). *$p < 0.05$ compared to controls (no CSE treatment). (B) THP-1 cell-derived macrophages without MARCO antibody incubation (mouse IgG isotype control) did not show fluorescent staining. Macrophages without CSE treatment (C) demonstrated stronger MARCO staining than those with 20% CSE (D) treatment. Original magnifications, x200.

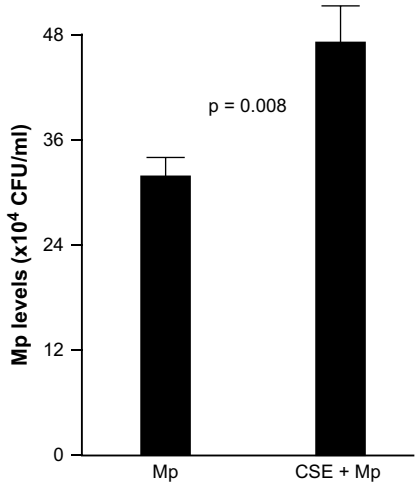

Figure 3 Pre-treatment of THP-1 cell-derived macrophages with cigarette smoke extract (CSE) impaired the clearance of *Mycoplasma pneumoniae* (Mp). Cells were exposed to cell culture medium (control) or 20% CSE for 6 h, and then infected with Mp (1 CFU/cell) for 2 h. Supernatants were collected for Mp culture. Mp levels in supernatants from CSE pre-treatment (CSE + Mp) cells were significantly higher than those in supernatants from cell culture medium pre-treated cells (Mp). Data are expressed as mean ± SEM ($n = 6$).

neutralizing antibody at 0.02 μg/ml reached its plateau in inhibiting Mp clearance.

To explain the blocking effects of MARCO neutralizing antibody on Mp clearance, we co-localized MARCO and Mp on THP-1 cell-derived macrophages. Briefly, macrophages on 8-well chamber slides were incubated with yellow fluorescent protein (YFP)-tagged Mp (1 CFU/cell)[29] for 2 h, and then fixed in methanol for double immunofluorescent staining of MARCO and YFP by using a monoclonal anti-MARCO antibody and a rabbit polyclonal anti-YFP antibody (Abcam Inc., Cambridge, MA), followed by FITC-conjugated anti-mouse IgG and Texas Red-conjugated anti-rabbit IgG. Fig. 5 demonstrates co-localization of MARCO with Mp on THP-1 cell-derived macrophages.

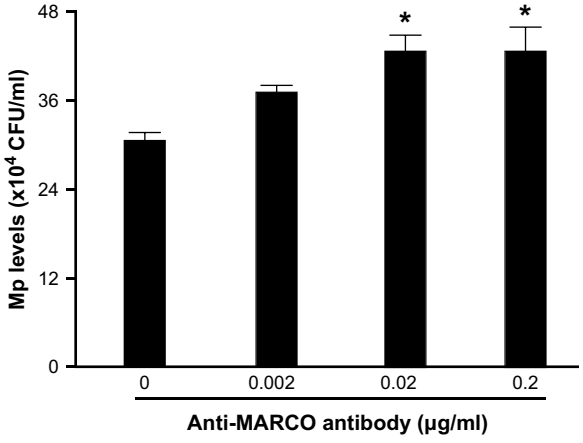

Figure 4 Blocking MARCO with a neutralizing anti-MARCO antibody increased *Mycoplasma pneumoniae* (Mp) levels in supernatants of THP-1 cell-derived macrophages. Anti-MARCO antibody was added to macrophages 1 h prior to Mp (1 CFU/cell) infection. At 2 h post-Mp, cell supernatants were collected to perform Mp culture. The results were expressed as mean ± SEM, $n = 4$. *$p < 0.05$ compared to cells without MARCO antibody treatment (mouse IgG isotype control).

Discussion

In this study we found that CSE down-regulated MARCO expression in human macrophages at both mRNA and protein levels. This decreased MARCO expression may be partially responsible for impaired clearance of Mp in that a neutralizing antibody against MARCO significantly impaired Mp clearance. Our data, for the first time, have provided evidence for a role of MARCO in host defense against bacterial (i.e., Mp) infection in a cigarette smoke exposure environment.

How MARCO is engaged in Mp clearance remains uncertain. It is possible that MARCO may directly bind to Mp, thus mediating the phagocytic process since the treatment with the MARCO neutralizing antibody resulted in impaired Mp clearance in macrophages. Further, MARCO was shown to be co-localized with Mp in our experiments. Our results are supported by an early study indicating a direct binding of MARCO to typical bacteria or their structural components including LPS.[30,31] A previous study also suggests that MARCO recognizes uteroglobin-related protein 1 (UGRP1), a secretory protein from airway epithelial cells that can directly bind to bacteria.[32] Thus, in the lungs, UGRP1 could serve as an additional mechanism to bridge Mp and MARCO to promote bacterial (e.g., Mp) clearance by macrophages.

It is not clear whether CS may increase the susceptibility of COPD patients to bacterial infections through inhibiting surface receptors such as MARCO. In our study, CSE was found to down-regulate MARCO expression in a dose-dependant manner. The down-regulation of MARCO on the surface of macrophages was accompanied by significant impairment of Mp clearance after Mp infection. Our findings suggest that CS may inhibit the ability of innate immune cells (i.e., macrophages) to eliminate the invading pathogens, thus leading to an increased load of bacteria in the lungs of COPD patients.

We realize that there are other scavenger receptors such as SR-AI/II, scavenger receptor with C-type lectin (SRCL) and SCARA5 receptor, which may also contribute to non-opsonised phagocytosis of pathogens (e.g., Mp) by macrophages. Indeed, SR-AI/II was shown to mediate phgocytosis of *Streptococcus pneumoniae* in mice.[33] Additionally, our current study has not revealed the detailed molecular mechanisms by which CSE suppresses macrophage MARCO expression. We propose that CSE may decrease MARCO expression in macrophages by altering inflammatory cytokines. For example, hydroquinone, a major metabolite of benzene present in large quantities in cigarette tar, was shown to inhibit production of IL-12 (a Th1-driving cytokine) in both primary mouse macrophages and RAW164.7 monocytic cells.[34] Interestingly, Jozefowski and co-workers demonstrated up-regulation of MARCO expression in J774 cells in the presence of IL-12.[35] However, our data indicate that after 24 h of Mp infection, IL-12 p70 levels were not different between THP-1 cell-derived macrophages with and without a 6 h CSE pre-treatment. Therefore,

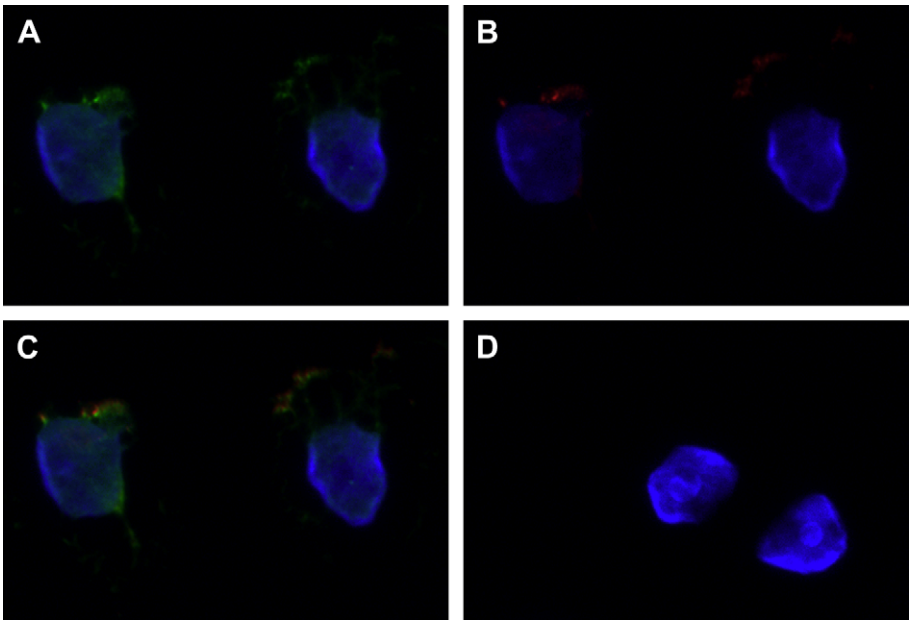

Figure 5 Co-localization of MARCO with *Mycoplasma pneumoniae* (Mp) on THP-1 cell-derived macrophages. Cells on 8-well chamber slides were incubated with yellow fluorescent protein (YFP)-tagged Mp (1 CFU/cell) for 2 h, and then fixed in methanol for double immunofluorescent staining of MARCO and YFP by using a monoclonal anti-MARCO antibody and a rabbit polyclonal anti-YFP, followed by FITC-conjugated anti-mouse IgG and Texas Red-conjugated anti-rabbit IgG. (A) Mp (green color) on the surface of THP-1 cell-derived macrophages. Blue color (DAPI staining) indicates cell nucleus. (B) MARCO staining on the same cell as shown in (A); (C) overlay of (A) and (B) demonstrating co-localization of Mp and MARCO. (D) cells incubated with mouse and rabbit control IgG (no MARCO and YFP antibody incubation) did not show any immunofluorescent staining. Original magnifications, x400.

mechanisms responsible for CSE-induced reduction of MARCO remain to be determined in our future experiments.

Since Mp can be detected in COPD patients,[2,3] we examined the impact of CS on Mp clearance. We found that CS decreased Mp clearance. Our findings have extended those from Green and colleagues in that CSE caused a dose-dependent inhibition of bacterial clearance by alveolar macrophages.[36] Here, we found that CSE reduced MARCO expression and then impaired Mp clearance. Together, our results have provided a novel pathway to explain an increased bacterial burden in lungs of smokers. That is, cigarette smoke reduces macrophage levels of receptors (e.g., MARCO) that are required in host innate immune responses to the invading bacteria, leading to dampened bacterial clearance. We are aware that our research findings in THP-1 cell-derived macrophages may not apply to all types of macrophages. To further support the relevance of our research findings in THP-1 cell-derived macrophages to human alveolar macrophages, we recently collected alveolar macrophages from bronchoalveolar lavage (BAL) cells of two normal healthy human subjects. Alveolar macrophages were obtained by adhering the BAL cells to 12-well tissue culture plates for 1 h at 37 °C. After removing the non-adherent BAL cells, adherent alveolar macrophages were collected to measure MARCO mRNA levels (baseline). The remainder of cells was exposed to 20% CSE for 6 h as described for THP-1 cell-derived macrophages. Our preliminary results suggest that like THP-1 cell-derived macrophages, human alveolar macrophages demonstrated reduced (about 1.7-fold reduction) MARCO mRNA expression after 6 h of exposure to 20% CSE. In our future studies, we will recruit more human subjects to obtain alveolar macrophages from non-smokers as well as smokers with or without COPD to evaluate MARCO expression levels, and to further define a role of MARCO in bacterial clearance in cigarette smoke-exposed alveolar macrophages.

Our study is an important step towards a better understanding of MARCO's role in bacterial infections in the context of cigarette smoke exposure. Our findings will be beneficial to future studies aimed at unraveling a role of lung macrophages in host defense against bacterial infections in individuals who smoke.

Conflict of interest

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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